Journal Homepage: -www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI:10.21474/IJAR01/7897
DOI URL: http://dx.doi.org/10.21474/IJAR01/7897
ISSN NO. 2320-5407

*RESEARCH ARTICLE*

# THE EFFECT OF FOOD QUANTITY ON PHYSIOLOGICAL RESPONSES OF THE BEARDED HORSE MUSSEL *MODIOLUS BARBATUS*AND THE NOAH'S ARK SHELL *ARCA NOAE.*

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## *Manuscript Info*

***Manuscript History***
Received: 12 August 2018
Final Accepted: 14 September 2018
Published: October 2018

***Keywords:***
*Arcanoae*, *Modiolusbarbatus*, oxygen consumption, ammonia excretion, clearance rate, food quantity

## *Abstract*

The aim of the work was to investigate oxygen consumption, ammonia excretion, *O:N* ratio derived from oxygen consumption and ammonia excretion, and the clearance rate of the bearded horse mussel *Modiolusbarbatus*(Linnaeus, 1758) and the Noah's Ark shell *Arcanoae*Linnaeus, 1758with regard to different food availability (100%, 50% and starving) during a fifteen day experiment. In this study, food quantity significantly affected ammonia excretion and O:N ratio for both species. Time spent under feeding regime resulted to increasing oxygen consumption and clearance rate values of the bearded horse mussel and the ammonia excretion values and O:N ratios of both species. Clearance rate and ammonia excretion followed a similar increasing trend over time. The consequence of increasing ammonia excretion over time was the concomitant reduction of the O:Nratio.Both species showed high values of clearance rate, an important information when choosing the farming site. Metabolic cost of respiration for starving animals was higher for *M. barbatus* than for *A. noae*, which suggests that *A. noae* would be a better choice for farming in oligotrophic environments.



## Introduction:-

Bearded horse mussel *Modiolusbarbatus*(Linnaeus, 1758)and the Noah's Ark shell *Arcanoae*Linnaeus, 1758have been an important fishery product in Mediterranean coastal areas since ancient Greek times (Voultsiadou et al., 2010). Recently these species were considered as aquaculture candidates on the eastern coast of the Adriatic Sea, so an aquaculture assessment was performed for both of them (Peharda et al., 2013; Županet al., 2014). Among other information, data on the impact of food quantity, especially the impact of starvation, on the physiology of shellfish is important for the selection of the farming site.

The influence of food quantity on oxygen consumption and the ammonia excretion was investigated on clam *Ruditapesdecussatus*(Khalil, 1994), banded carpet shell *Polititapesrhomboides* and dog cockle *Glycymerisglycymeris* (Savina and Pouvreau, 2004), clams *Ruditapesdecussatus*and the pullet carpet shell*Venerupiscorrugata* (Albentosaet al., 2007). There are numerous studies about the influence of algae

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concentration on the clearance rate of the shellfish (e.g. Clausen and Riisgård, 1996; Fernández-Reiriz et al., 2005; Nieves-Soto et al.,2013). The influence of temperature on physiological responses (Egzeta-Balićet al., 2011) was investigated for the bearded horse mussels. There are many studies on physiological responses of *M. barbatus* with regard to stress caused by temperature changes asmetabolic and molecular responses to stress (Anestis et al., 2008; Katsikatsou et al., 2009, 2010), biochemical and cellular response to stress (Dimitriadis et al., 2012), climate change impact (Katsikatsou et al., 2012), and the interaction of heavy metals and temperature (Katsikatsou et al., 2011). Differences in physiological responses due to differences in body size have been documented for *A. noae* and *M barbatus* (Glavić et al., 2018).

The aim of this paper was to investigate physiological responses of oxygen consumption (OC), ammonia excretion (AE) and the clearance rate (CR) for *M. barbatus* and *A. noae*, reared at different feeding regimes, including food deprivation. In addition, the effect of duration of feeding regime on physiological responses was studied. Physiological responses were also compared between species. Since there are no available data on the physiological responses to different food quantity for these species, we deliver a new contribution in overall knowledge important for their farming.

## Materials And Methods:-

**Sampling and experimental design**

Individuals of *M. barbatus* and *A.noae* were collected by autonomous diving in May 2014, May 2015 and May 2016 in Bistrina Bay (42.869836° N, 17.701869° E) (Fig 1) within the Mali Ston Bay. The studies of OC and AE were performed on the individuals collected in May 2104 and 2015 while the study of CR was perfromed on the individuals collected in MAy 2016. Bivalves were transferred to the laboratory of the Institute for Marine and Coastal Research in Dubrovnik. The acclimation period of shellfish to the aquarium conditions lasted for 7 days. During this period, shellfish were fed daily with the flagellate *Tetraselmissuecica* in a density of $3x10^3$ cell $ml^{-1}$. Determined food quantity was approximately 0.43 mg $l^{-1}$ ($3x10^3$ cell $ml^{-1}$) according to Widdows and Staff (2006) recommendation.

Measurements of OC, AE and CR were carried out in relation to 3 different feeding regimens: 100% and 50% daily feed, and starvation (no food), where 100% daily feed is flagellate *T. suecica* at levels $3x10^3$ cells $ml^{-1}$ (Widdows and Staff, 2006). We have assumed that selected amount of foodof $3x10^3$ cell $ml^{-1}$ was sufficient for survival of the studied shellfish and the best choice to investigate the influence of the amount of food and the effect of feeding time on the physiological responses of the studied species. *T. suecica* is large and rich in proteins (Brown, 1991). Mussel Mytilusedulis showed a positive Scope for growth if fed with concentrations *T. suecica* greater than 2500 mL-1 (Thompson and Bayne 1974).

Physiological responses were measured on the first, fifth, tenth and fifteenth day. CR of animals was measured concomitantly to reveal the dependence of their energy needs on the amount of food available. The experimental bivalve molluscs were divided into three groups according to the feeding regime. From the group of 30 market sized individuals (40-50 mm), five for each feeding regime were monitored on a daily basis. OC measurement was performed at the end of the period between two feedings to avoid the effect of feeding (Widdows and Staff, 2006). OC and AE were measured for each animal in the closed respiratory chamber (volume 429.39 ml). The experiment was conducted at temperature of 18 ± 1˚C and salinity of 35 ± 1 psu. Prior to measurement the shellfish were cleaned off from fouling. For the first 30 minutess animals were kept in the chamber with flow through of seawater in order to adapt to chamber conditions, opening of the shell and start of ventillation. After that, for the next 40 min the values of dissolved oxygen (DO) were read at intervals of five minutes. The DO concentration drop was measured by the Oxyscan graphic probe (UMS Gmbh, Germany). No significant drop in DO was observed in the control chamber without shellfish. DO spent during one hour of experiment i.e. $VO_2$ (mg $O_2$ $h^{-1}$) was calculated according to Widdows and Johnson (1988) by the formula: $VO_2 = 60 \times \left[ C(t_0) - C(t_1) \right] \times (Vr) / (t_1 - t_0)$

where: $t_0$ and $t_1$ are the initial (min) and the final time (min) of the measurement period, $C(t)$ is the oxygen concentration in water at time $t$, $Vr$ is the volume of respirometry chamber reduced by volume of shellfish. After oxygen measuring period, 50 ml of the sea water sample was taken from the closed chamber to measure total ammonia concentration. Daily controls of total ammonia concentration are subtracted from the values obtained in the experimental chamber to calculate AE rate. The concentration of ammonium/ammonia i.e. total ammonia was

determined by the indolfenol blue method (Solorzano, 1969) with modification to Ivančić and Degobbis (1984). The ammonia excretion rate was calculated according to Sobral and Widdows (1997) by the formula:

$$U = (T - C) \times (V/1000)/t$$

where: $U$ – ammonia excretion rate (μmol $NH_4$-N $h^{-1}$), $T$ - concentration of ammonia in the sample (μM) , $C$ - concentration of ammonia in the control sample (μM), $V$ - volume (ml) of the seawater in which the animal is immersed, $t$ - time (h). The atomic ratio of oxygen and nitrogen (O:N) was determined on the basis of OC and AE.

Energetic expenditures are respiratory energy expenditure (R) and energy lost in excreta (U). Calculation of R and U (all in J $g^{-1}$ $h^{-1}$) is according to Widdows and Johnson (1988) as follows:

$$R = VO_2 \left( ml\ O_2 g^{-1} h^{-1} \right) \times 20.33\ J\ ml^{-1} O_2$$

$$U = mg\ NH_4 g^{-1} h^{-1} \times 19.4\ J\ mg^{-1} NH_4 .$$

For the measurement of CR, bivalves were cleaned from fouling, placed in a container, left for 20 minutes to open the shells and then the algal cells (*T. suecica*) were added. The algal cell density was measured in the tank half an hour after the addition of $20x10^3$ cell $ml^{-1}$, which was repeated at 60, 90 and 120 min. The CR, which is the volume of water cleared from suspended particles in the unit of time, was calculated from the exponential drop in algal cell concentration in the water tank over a period of 1.5-2 hours (Widdows and Staff, 2006). Mixing of water was achieved by aeration. No significant drop in the cell abundance was observed during the experimental period in the control tanks without shellfish. CR measurements were carried out in the containers of a volume of 5 l. CR of each bivalve was calculated according to Coughlan (1969):

$$CR\left( l\ h^{-1} \right) = \mathrm{Vol}(l) \times \left( \ln C1 - \ln C2 \right) / time\ interval\ \ (h)$$

where: *Vol* is the volume of water, $C_1$ and $C_2$ are the cell concentrations at the beginning and the end of measuring, respectively. Measurement of algal culture density was performed using the Hach DR2500 spectrophotometer at λ=750 nm. Regression analysis of the dependence of absorbance on known population density yielded the calibration curve, and subsequently, polynomial equation of that regression was used to calculate population density from absorbance data (Rodrigues et al.,2011). In order to standardize the values of physiological rates and eliminate the influence of different animal weight, all physiological rates were converted to a specific physiological rate per gram of dry mass of the animal. After the experiment, the soft tissue of the bivalve was dried in a drying chamber at 60°C for 24 h to a constant mass and weighted. The dry shellfish weight was taken as the basis for calculating the specific physiological rate according to Bayne and Newell (1983) using the following equation:

$$Ys = (Ws/We)^b \times Ye ,$$

where: *Ys* is the physiological rate for the animal of standard mass (1 g), Ws is the standard mass (1 g), *We* is the observed mass of the individual in grams, *Ye* is the uncorrected physiological rate, b is the mass exponent for the physiological rate. The mean b exponent of mass is 0.67 for CR and 0.75 for OC of bivalve (Savina and Pouvreau, 2004), and a value of 0.78 was used for AE according to Hawkins et al. (2000).

**Statistical analysis**

The data collected were tested for the homogenity of variance using Levene's test using the Statistica package 12.0 (Statsoft Inc.). Normality of data was estimated by Kolmogorov-Smirnov and Liliefors test. Upon confirmation of normal distribution of values within groups, further analysis was carried out using parametric ANOVA and Tukey post-Hoc analysis. For the analysis of OC, AE, O:N ratio and CR with regard to different food quantity and feeding time of bivalves, nested ANOVA ($p < 0.01$) was used. To compare the metabolic rates between species *A. noae* and *M. barbatus*, a t-test was used.

## Results:-

**Oxygen consumption**

Mean OC for *M. barbatus* depended significantly (ANOVA, $p < 0.01$) on feeding time. Mean values ofrespiration rate for *A. noae* were from 0.20 ± 0.09 to 0.46 ± 0.16 $mgO_2g^{-1}h^{-1}$and energy loss due to respiration from 2.80 ± 1.28 to 6.55 ± 2.28 $Jg^{-1}h^{-1}$. Mean values of respiration rate for *M. barbatus* were from 0.25 ± 0.09 to 0.59 ± 0.28$mgO_2g^{-}$

$^{1}h^{-1}$and energy loss from 3.56 ± 1.28 to 8.40 ± 3.98$Jg^{-1}h^{-1}$.*M. barbatus* showed higher metabolic cost of respiration than *A. noae*for starving animals (Fig 2). T-test showed a statistically significant difference for respiration rate between these two species for shellfish fed 10 days with 50% of food (t = -3.96, $p$ = 0.004) and fifteenth days of starving (t = -2.94, $p$ = 0.018).
Fig 2

**Ammonia excretion**
AE rate of both *A. noae* and *M. barbatus* depended significantly (ANOVA, $p < 0.01$) on food quantity and time of feeding (ANOVA, $p < 0.01$). Mean values of AE for *A. noae* were from 0.56 ± 0.26 to 4.46 ± 1.06 $\mu molg^{-1}h^{-1}$, and energy loss due to excretion from 0.20 ± 0.09 to 1.56 ± 0.37 $Jg^{-1}h^{-1}$. Post hoc Tukey test for *A. noae* revealed significant difference between groups of bivalves starving for 5, 10 and 15 days and all groups fed with 100% food (Tukey, $p < 0.01$). There was also difference between groups fed 1, 5 and 10 days with 50% food and starved group for 15 days. Mean values of AE for *M. barbatus* were from 0.84 ± 0.17 to 3.89 ± 0.82 $\mu molg^{-1}h^{-1}$, and energy loss due to excretion from 0.29 ± 0.06 to 1.36 ± 0.29 $Jg^{-1}h^{-1}$. Post hoc Tukey test for *M. barbatus*revealed significant difference between groups of bivalves starving for 15 days and all groups fed with 100% food (Tukey, $p < 0.01$), 15 days starving and 1 and 5 days fed with 50% of food (Tukey, $p < 0.01$) and also between group 1 day and 15 days starving (Tukey, $p < 0.01$). Metabolic cost of excretion is higher for *A. noae* than *M. barbatus* for starving animals (Fig 3). T-test did not show a statistically significant difference for AE rate between *A. noae* and *M. barbatus.*
Fig 3

**O:N ratio**
O:N ratio of both investigated species significantly depends on food quantity (ANOVA, $p < 0.01$) and time of feeding (ANOVA, $p < 0.01$) and starved animals had the lowest ratio (Fig 4). Mean values of O:N ratio for *A. noae* were from 2.89 ± 0.98 to 30.90± 13.01. The post hoc Tukey test howed a significant difference for *A. noae* between all groups without food and a group one day fed with 100% food; between group one day fed with 100% and other groups fed with 100% food; between group one day fed with 100% and groups fed 5, 10 and 15 days with 50% food (Tukey, $p < 0.01$). Mean values of O:N ratio for *M. barbatus* were from 6.81 ± 1.92 to 25.98 ± 8.73. The post hoc Tukey test howed a significant difference for *M. barbatus*between groups starved 5, 10 and 15 days and group fed 5 days with 100% food (Tukey, $p < 0.01$). T-test showed a statistically significant difference for O:N ratio between *A. noae* and *M. barbatus* for the fifth day of starving (t = -4.19, $p$ = 0.003), for the fifteenth day of starving (t = -3.96, p = 0.004), for the tenth day of feeding with 50% of food (t = -2.59, $p$ = 0.032) and the fifth day of 100% feeding (t = -2.55, $p$ = 0.034).
Fig 4

**Clearance rate**
CR depended significantly on feeding time for *M. barbatus*(ANOVA, $p < 0.001$). Mean values of CR were for *A. noae* from 3.75 ± 0.16 to 9.89 ± 4.47 $lh^{-1}g^{-1}$, and for *M. barbatus*from3.41 ± 0.85 to 10.34 ± 4.08 $lh^{-1}g^{-1}$. For both species CR increased over time (Fig 5). The post hoc Tukey test showed a significant difference for *M. barbatus*between groups of bivalves 1, 5 and 10 days without food and 15 days starving group; between 15 days starving group and 1 and 5 days fed with 100% (Tukey, $p < 0.01$). T-test showed that there was not a statistically significant difference for CR between these two species.
Fig 5

## Discussion:-
Prior to undertaking efforts in aquaculture, it is important to understand the influence of environmental factors variation on the physiology of cultivated organisms. Data on physiological responses are very important for the management of bivalve molluscs culture. Metabolic studies under limited food availabilty help to increase our knowledge on physiological mechanisms emplyed by the organism to overcome such periods (Bayne, 1973a).

**Oxygen consumption**
The values of OC i.e. respiration rate for explored bivalves of both species are mostly consistent with data obtained on several occasions (Hawkins et al., 1985; Widdows et al., 1990; Savina and Pouvreau, 2004). Some researchers also noticed no influence of food quantity on OC (Hawkins et al., 1985; Savina and Pouvreau, 2004). Laboratory experiments on *M. edulis* showed that OC decreased during period of 80-90 days, with a higher drop in the unfed animals than in the fed animals (Bayne and Thompson, 1970). Decrease in the rate of respiration was reported for starved clams after 15 days (Albentosaet al., 2007). In our research, the OC was measured for 15 days, so it is

possible that *M. barbatus* and the *A. noae*needed more time to demonstrate the effect of food quantity. The starved *A. noae*, as expected, showed a similar trend of reduction in respiration rate (Bayne, 1973a), though not statistically significant ($p > 0.01$). For *M. barbatus*, the opposite occured that 15 days of starving resulted in a significant increase in OC. *M. barbatus* starving 15 days showed two times higher metabolic cost than *A. noae* in same conditions. Higher metabolic cost of respiration of *M. barbatus* was observed in comparison to *A. noae* (Glavić et al., 2018). Species inhabiting shallow coastal waters have more food and a less need for respiratory adaptation (Newell and Branch, 1980), what is case with Mali Son Bay (Meštrov and Požar-Domac, 1981) so both investigated species probably did not need physiological adjustment on the amount of given food over the duration of the experiment.

**Excretion rate**

The high excretion rates observed in both species under starvation were consistent with the studies of blue mussel *M. edulis* (Bayne and Thompson, 1970; Bayne, 1973a,b). Using proteins as energy substrate during this period of the year (Bayne and Scullard, 1977b) resulted in high values of AE. Increased AE was lasted the first 30-40 days of starvation and then decreased (Bayne, 1973b). An increased excretion of starved clam *R. decussatus* was noticed in comparison with those fed at 16° C, but not at 20° and 28°C (Khalil, 1994). The values of AE in this research were consistent with the results reported in some studies (Khalil, 1994; Guzmán-Agüero et al., 2013), but much lower values were documented as well (Hawkins et al., 1985; Stead and Thompson, 2003).

High values of ammonia excretion were observed in shellfish collected from brackish water in Vrångskär area (Gilek et al., 1992) as Mali Ston Bay. Differences in values, except for different types of shellfish, might be associated to the different period of the year during the experiment and the use of different types of food. The effect of the flagellate *T. suecica* to increased ammonia excretion was observed (Bayne and Scullard, 1977a). Authors found an increase in AE 24 to 30 hours after the feeding in experiments in which the mussel *M. edulis* were fed with flagellate *T.suecica*. Combination of influence of season (the highest values of AE in spring and summer) (Widdows, 1978) and type of food (algae *T. suecica)* resulted in exceptionally high ammonia value. AE was shown also to be very difficult to estimate in a relation to the feeding conditions because of the stress caused by the collecting of animals which might have contributed to the fluctation of the AE (Ikeda, 1977).

O:N ratio

O:N ratio reflects the ratio of proteins to lipids and carbohydrates that are degraded as energy substrates (Navarro and Thompson, 1996). In our experiment the values of O:N ratio for *A. noae* were under 30 indicating the use of proteins as a metabolic substrate except for bivalves fed one day with 100% of food. The lowest values have been achieved for starved animals, suggesting they were under the greatest stress. All recorded values of O:N for *M. barbatus* were less than 30. Our experiment was carried out in the late spring, there was a possibility that the females preserved fats because of the preservation of gamete material for being in ripe stage of sexual maturation (Peharda et al., 2006), while carbohydrates and proteins were used as a metabolic substrate (Albentosaet al., 2007). Having a low quantity of carbohydrates in the late spring (Bayne, 1973b) bivalves probably used proteins and this could explain low values of O:N ratio of both species.

**Clearance rate**

In this study the value of CR for the *A. noae* and *M. barbatus*overlaped and are in accordance with the values observed in some studies (Widdows and Johnson, 1988; Widdowset al., 1990). In the research of influence of temperature on physiological responses the much lower values of CR were reported for *M. barbatus*(0.17 to 0.23 $lh^{-1}g^{-1}$) (Ezgeta-Balić et al. 2011). Bivalves in that research were fed with a larger amount of algae *Isochrysisgalbana*, and experiment was conducted in November, while our research was conducted at the end of May. Due to the whole set of differences, and the lack of information on the cumulative effect of different impacts, it was difficult to compare results.

In both investigated species the same trend of significantly increasing CR with time was present. For the scallop *A. purpuratus* was observed a significantly higher CR after three days of acclimation in a low-food regime (1.03 $mgl^{-1}$) (Fernández-Reirizet al.,2005), so it was possible that in our work the time has ifluenced the CR of investigated bivalves. Similar to our research, no influence of food concentration on bivalves was noted for the dog cockle *G. glycimeris*(Savina and Pouvreau, 2004). This was in agreement with finding that CR should be independent of the concentration of particles until a certain threshold (Sprung and Rose, 1988). For *M. barbatus* fifteen days of starving results in a significant increase in CR.

## Conclusions:-

During fifteen days of starvation, *M. barbatus* OC and CR showed an increase. Possibly in that way it coped with a lack of food by increased filtration, and in turn by higher respiration. It would be desirable to investigate what is happening with *M. barbatus* starving more than 15 days.

It is assumed that the increased clearance rate over time in fed animals and consequently the higher consumption of algae *T. suecica*, which contains large amounts of protein, can cause increased ammonia excretion as both (CR and AE) follow a similar trend. Since in fed animals OC is decreasing over time, the O:N ratio is a consequence of increasing AE.

With regard to the CR and AE there was no significant difference between these two species. As for the other physiolgical responses, the biggest difference between species was for the groups fed 10 days with 50% of food, and in the case of groups starving for 15 days.

Starvation affected excretion of hugh amount of ammonia in both species. This information should be considered when choosing a farming site in the case of intensive farming.Oligotrophic areas might be susceptible to the increased levels of excreted ammonium since the phytoplankton community could react by blooms after receiving pulses of ammonium that supports its fast growth. Harmful algal blooms and its toxicity on human health via consumption of shellfish is a topic beyond the scope of our study.

For *M. barbatus* feeding time showed influence on all physiological responses suggesting importance of duration of unsuitable food conditions. High values of the CR for both species species represent an important information when assessing ecological carrying capacity and selecting the farming site. Metabolic cost of respiration for starving animals is higher for *M. barbatus* than for *A. noae*, so we suggest that *A. noae* would be a better choice for farming in the oligotrophic sea.

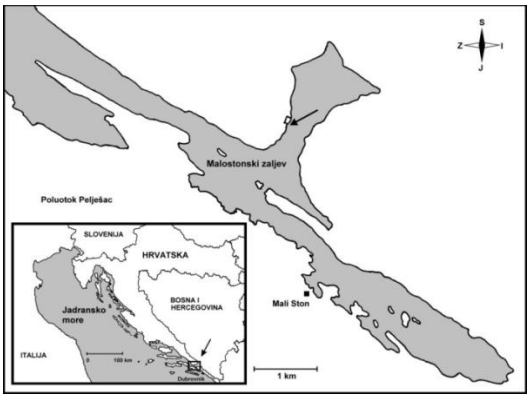


**Fig. 1:-**Location of collecting of *A. noae* and *M. barbatus* – *Mali Ston Bay*, collecting site is shown by an arrow.

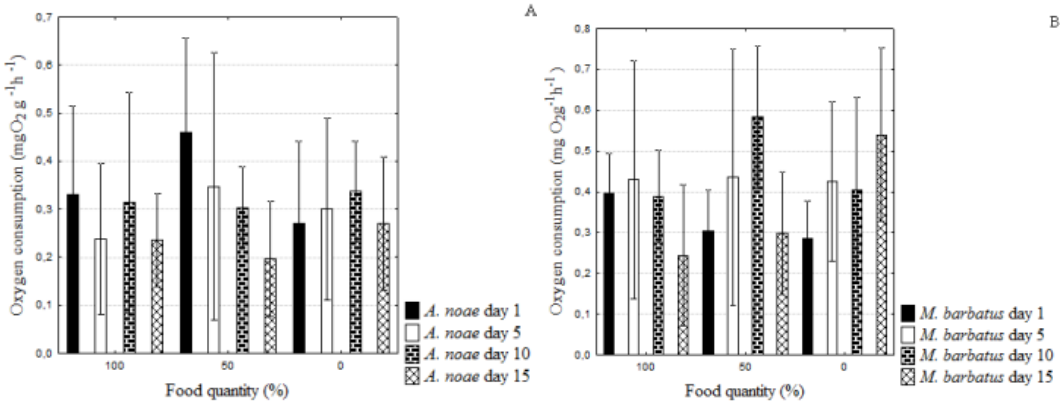


**Fig.2:-**Oxygen consumption ($mgO_2g^{-1}h^{-1}$) of *A. noae* (A) and *M. barbatus*(B) related to food quantity and feeding time. Concetration of algae *T. suecica* of 3000 cell $ml^{-1}$ was determined as 100% food quantity.

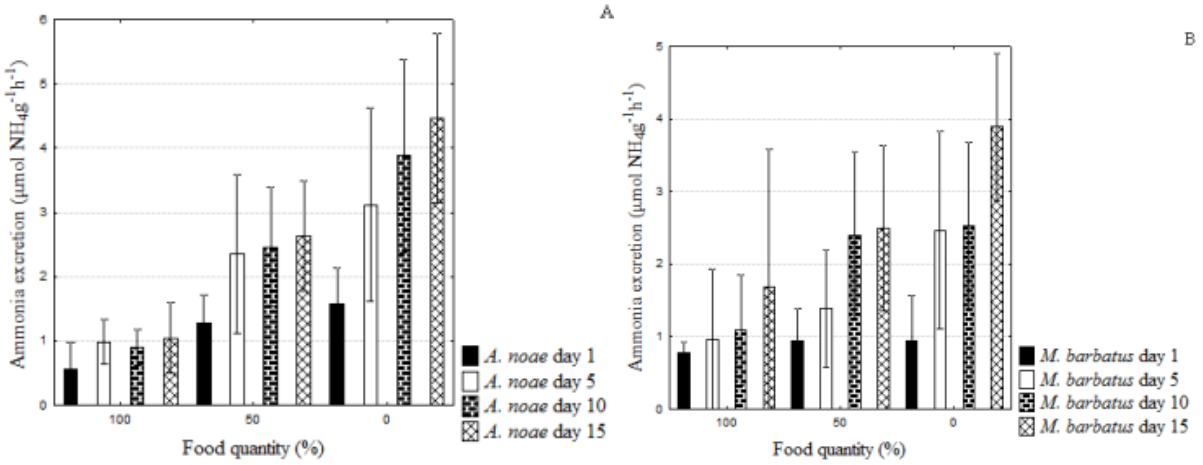


**Fig. 3:-**Ammonia excretion ($\mu molNH_4g^{-1}h^{-1}$) of *A. noae* (A) and *M. barbatus* (B) related to food quantity and feeding time. Concetration of algae *T. suecica* of 3000 cell $ml^{-1}$ was determined as 100% food quantity

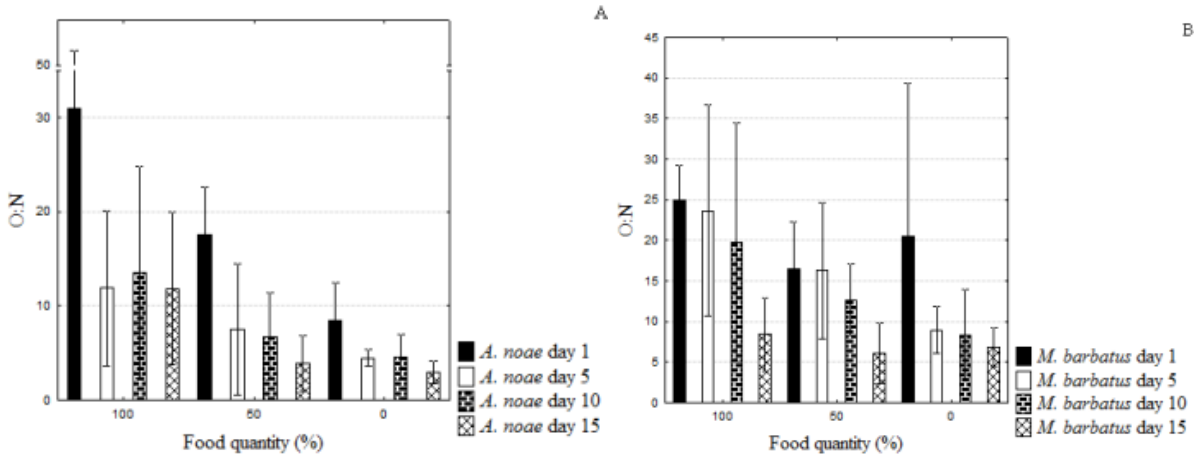


**Fig.4:-**O:N ratio of *A. noae* (A) and *M. barbatus* (B) related to food quantity and feeding time. Concetration of algae *T. suecica* of 3000 cell $ml^{-1}$ was determined as 100% food quantity.

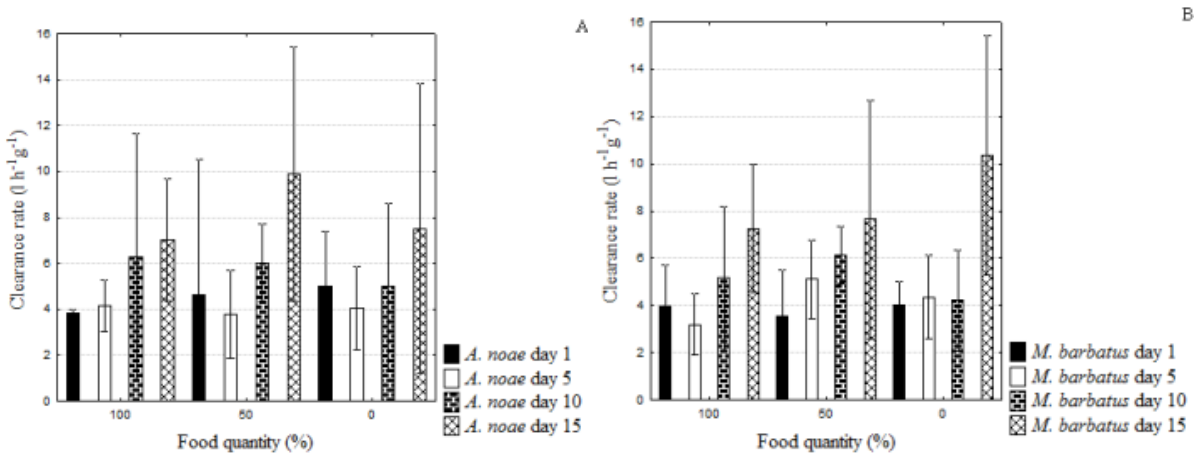


**Fig. 5:-**Clearance rate ($lh^{-1}g^{-1}$) of *A. noae* (A) and *M. barbatus* (B) related to food quantity and feeding time. Concetration of algae *T. suecica* of 3000 cell $ml^{-1}$ is determined as 100% food quantity.

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